INVESTIGATION OF THE CONFORMATION, SOLUBILITY, STRUCTURE AND SELECTIVITY OF ALKALINE PROTEASES IN ANHYDROUS ALCOHOLS.

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Summary

The solubility of two alkaline protease, alcalase and subtilisin Carlsberg, in absolute alcohol are determined. The global conformation as well as the local conformation of the active sites (S-1 and S-2 subsites) of both enzymes in alcohol and in aqueous solution are similar. The enantio- and diastereo-selectivity of both enzymes in anhydrous alcohol are also similar to those in aqueous solution.

Recently, the search for proteases that are stable in organic solvents for peptide synthesis has been extensive.[1] Several studies have demonstrated that it is possible to use proteases to catalyse peptide synthesis in organic solvents.[2] Some enzymes can maintain selectivities in organic solvents, and others cannot.[3] This report describes a study of solubilities, selectivities, and conformations of two alkaline proteases, alcalase and subtilisin Carlsberg, in anhydrous alcohol. The solubility of both enzymes in anhydrous absolute alcohol was determined by Coomassie Blue method using BSA as a reference. The CD and UV spectra were used to investigate the global conformation of the enzymes in alcohol and the state of enzymes in alcohol. Selective transesterification of substrates, Cbz-DL-amino acid methyl esters and Bz-DL-Ala-L-Phe-OMe, in anhydrous absolute alcohol were used to study the local conformation of the active sites (S-1 and S-2 subsites) of the enzymes in anhydrous alcohol.[4] A procedure for preparing active enzymes in anhydrous alcohols has been reported.[5]

Alcalase is a proteolytic enzyme prepared from the submerged formation of a selective strain of *Bacillus licheniformis*. The major enzyme component of alcalase is the subtilisin Carlsberg (alkaline protease A), which is an extracellular protease and is commercially available as a brown liquid.[6] Pure alcalase was obtained via dialysis in phosphate buffer to remove the nutrition broth, precipitation by adding isopropanol , and purification by gel filtration on TSK HW-50S gel. Commercial subtilisin Carlsberg (Sigma, Protease VIII, obtained with 11.6

U.mg⁻¹) was purified to a homogeneous state via TSK HW-50S gel filtration. The alcalase contained a small

amount of denatured enzyme, and the subtilisin Carlsberg contained lower MW peptides. The fractions of pure

enzyme were collected and used directly (alcalase, 23.7 $U.mL^{-1}$; subtilisin carlsberg 21.4 $U.mL^{-1}$) .

Figure 1-a shows the UV spectrum of the alcalase in phosphate buffer and in absolute alcohol, and the

denatured alcalase in buffer. The UV spectrum was obtained by measuring the absorbance (360–200 nm) of a

solution that was prepared by adding 100 μL of freshly prepared enzyme solution to 3 mL of buffer or absolute

alcohol, respectively. The patterns of the three spectra are identical. Equal amounts of the enzyme had the same

UV absorbance no matter whether
in aqueous solution or in absolute
alcohol. The denatured alcalase
was nearly insoluble in absolute
alcohol. In same manner, the UV
spectra of pure subtilisin Carlsberg
in absolute alcohol was measured
and the results are shown in Figure
1-b. The UV spectrum of subtilisin
Carlsberg in absolute alcohol and
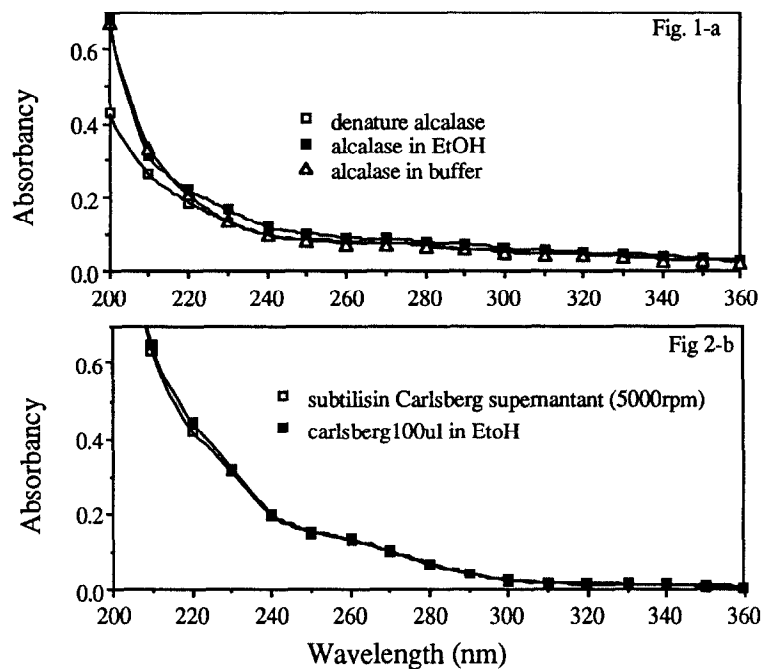in aqueous solution are idential
also.

Figure 1.   UV spectra of alcalase and subtilisin carlsberg. The UV spectra were measured by adding 100 μL
             of fresh eluted enzyme solution to a cuvette containing phosphate buffer (10 mM, pH 7.0) to make
             the final volume exactly 3.0 mL.

1-a.   Alcalase in ethanol, in buffer, and denatured alcalase in buffer. The denatured enzyme was
        prepared by adding methanol (5 mL) to the fresh prepared enzyme solution (2 mL) and incubating
        for 8 hours. The resulting solution was lyapholized and re-dissolved in water (2 mL). 100 μL of
        the solution was transferred to a cuvette for spectra measurement.

1-b.   UV spectra of subtilisin Carlsberg in absolute ethanol. The first spectrum was measured by adding
        100 μL of fresh eluted enzyme solution to ethanol as described above. The sample solutions of the
        other two spectra were prepared via precipitation of the freshly eluted enzyme by ethanol for three
        times, resuspension of the precipitated enzyme in ethanol (20 mL), anddivision of the resulting
        mixture into two portions, which were centrifuged for 5000 rpm . The superntant was transferred
        to a cuvette for UV spectra measurement.

Figure 2 shows the CD spectra of alcalase. The spectra were measured with the same concentration as that in the UV spectra measurement. The CD spectra of both enzymes in alcohol and in phosphate buffer (pH 6.2) are identical and are also identical to a reported spectrum.[7] The CD spectra of the denatured enzymes in buffer showed a random structure. The denatured enzyme was insoluble in absolute alcohol, and no CD spectrum was observed. The spectra results showed that the global conformation of the pure alkaline proteases was not changed in alcoholic solvents, and that the conformation of alcalase was similar to that of subtilisin Carlsberg. Measurement
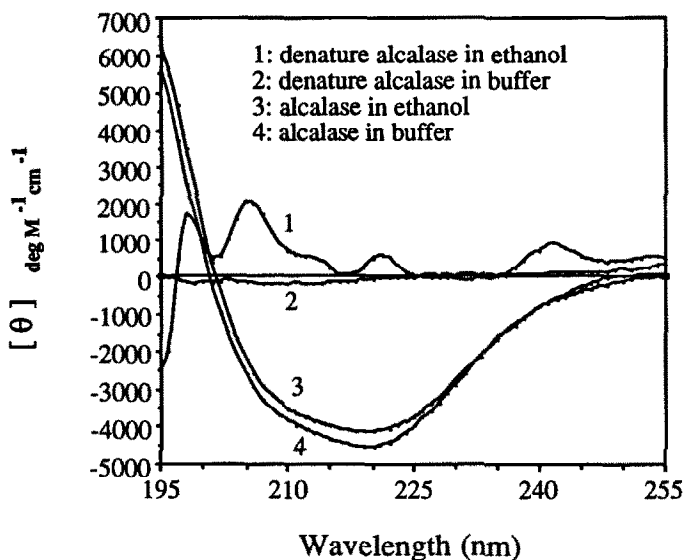
of the conformation of an active protease in organic solvent has been rare due to the fact that organic solvents have been used as precipitating reagents, and the protein was insoluble or would denature in these solvents. Both the absorbancy of UV and ellipticity of the CD spectra of the enzyme in alcohol were identical to that in aqueous solution indicate the state of the enzymes in alcohol and in aqueous solution is the same.

Figure 2. CD measurements were taken on a Jasco-720 spectropolarimeter, using cuvettes of 1 mm pathlength. Enzyme concentrations were 1.33×10^{-2} mg/mL.

For further investigation solubility of the enzymes in alcohol, a freshly eluted fraction containing active subtilisin Carlsberg was precipitated and centrifuged with absolute alcohol for three times to remove water.[5] The solubility of the subtilisin Carlsberg and alcalase in alcohol was determined by taking the supernantant and reacted with Coomassie Brilliant Blue G. The solubility of alcalase and subtilisin Carlsberg in absolute alcohol was 0.045mg.mL^{-1} and 0.048 mg.mL^{-1} respectively as measured by the absorbance at 595 nm using Bovine serum albumin as a reference standard.

Selective trans-esterification using the substrates Cbz-D,L-AA-OMe, (AA= Ala, Aba, NorVal, NorLeu, Phe) and Bz-DL-Ala-L-Phe-OMe catalyzed by alcalase and subtilisin Carlsberg in absolute alcohol was performed at 35°C using HPLC to monitor the reaction. The results are shown on table 1. The enzymatic activity was determined by measuring the increased concentration of the product in the first 1 min. After about 50% of the substrate was converted to the ethyl ester, the resulting mixture was evaporated, and the products were isolated

by preparative HPLC. The enantiomeric excess (e.e.) of the products was determined by a chiral HPLC column,

and the e.e. of both products ranged between 100% ~ 85%. The optical purity of the diastereoselective products

Bz-D-Ala-L-Phe-OMe and Bz-L-Ala-L-Phe-OEt was 98% and 91%, respectively, as measured by a silica gel

HPLC column. Both alcalase and subtilisin Carlsberg had high catalytic reactivity and selectivity in absolute

alcohol. The selectivity of alkaline potease catalyzed-transesterification in absolute alcohol was as high as the

reported for alcalase catalyzed hydrolysis in aqueous solution[8b,c] and in t-butanol containing 5% buffer.[8a] The

results indicated that the conformation of the active site (S-1 and S-2 subsites) of the enzymes in absolute alcohol

may be the same as that in aqueous solution.

Table 1. Selective trans-esterification catalyzed by alkaline protease in anhydrous ethanol.[a]

Substrates	enzyme	initial rate x10^{-3}	conversion	e.e. (%)	
		μm/min.unit.		methyl ester	ethyl ester
Cbz-Ala-OMe	alcalase	205.3	49	97 (97)[b]	100 (98)[b]
"	subtilisin Carlsberg	272.8	44	87	100
Cbz-Aba-OEt[c]	alcalase	450.7	49	97[c] (100)[b]	96[c] (97)[b]
Cbz-NorVal-OMe	alcalase	412.6	51	97 (100)[b]	97 (95)[b]
Cbz-NorLeu-OMe	alcalase	221.1	49	100 (96)[b]	91 (98)[b]
"	subtilisin Carlsberg	273.6	44	89	100
Cbz-Phe-OMe	alcalase	322.9	48	97 (98)[d]	93 (98)[d]
Bz-Ala-Phe-OMe	alcalase	-	46	91	98
"	subtilisin Carlsberg	-	44	90	100

a.Transesterification activity. To Cbz-DL-AA-OMe (for alcalase, 139 mg of Cbz-DL-Ala-OMe, 80 mg of
 Cbz-DL-Aba-OEt, 105 mg of Cbz-DL-NorVal-OMe, 138 mg of Cbz-DL-NorLeu-OMe, 102 mg of Cbz-DL-
 Phe-OMe was used; for subtilisin Carlsberg, 47 mg of Cbz-DL-Ala-OMe, 81 mg of Cbz-DL-NorLeu-OMe
 were used) dissolved in absolute ethanol (7 mL) was added a freshly prepared ethanol solution (3 mL)
 containing alcalase (1.0 AU) or subtilisin carlsberg (80 mg, 11.0 u/mg). The resulting solution was stirred at
 35°C. Periodically, aliquots of samples (0.2 mL) were taken and quenched by the addition of enough HCl
 (0.10 N, 0.8 mL) to make a final volume of 1.0 mL . The solution was centrifuged for 5 minutes at 3000 rpm,
 and aliquots (20 µL) of the supernatant were analyzed by HPLC using a RP-18 column, a uv detector at 254
 nm, and 1:1:0.01 (v/v/v) acetonitrile/water/trifluoruacetic acid as the eluent. The peak area corresponding to
 Cbz-AA-OMe was determined, and the reaction rates of transesterification were measured by fitting the area to
 a calibration curve for Cbz-AA-OMe. The reaction was continued until 50% of the conversion was reached
 (20-60 min), and then the mixture was quenched by the addition of HCl (1.0 N). The products were isolated
 by preparative HPLC under the same conditions. The enantiomeric excess of amino acid derivatives was
 determined by using a chiral column (Diacel Chiral WH, using eluent, ipa:n-hex 95:5). The optical purity of
 diastereomeric peptide was determined using a silica gel column (si 100, using IPA:n-Hex 90:10 as eluent).
b. The data was obtained by resolution in a solution of 5% buffer/95% t-butanol (ref. 8a).
c. The substrate was an ethyl ester of Aba, and the reaction was conducted in n-propanol solution;
 thus, the product was an n-propyl ester.
d. The data was obtained by resolution in aqueous solution with 30% acetone (ref. 8c).

The subtilisin Carlsberg was obtained as a white crystal, which was hard to dissolve in alcoholic solvents. The active subtilisin Carlsberg was prepared by dissolving the enzyme in buffer containing 1% $CaCl_2$ and treated using the same procedure as for alcalase.[5] The enzyme prepared in this way was two hundred times as active as the enzyme used in crystal form.

That the alcalase can maintain its enzymatic activity in alcoholic solvents has been documented.[3b,c] This study demonstrates that the alcalase and subtilisin Carlsberg catalyzed-reaction in anhydrous alcohol have the same selectivities as when catalyzed in aqueous solution or in 95% t-butanol containing 5% buffer. These results are opposite to those for the subtilisin Carlsberg catalyzed transesterification in other organic solvents.[3a,b] In conclusion, the conformation of subtilisin Carlsberg and alcalase in anhydrous alcohol is identical to that in aqueous solution.

Acknowledgment.

References.

1). a) C. H. Wong, K. T. Wang, Experientia, 1991, 47, 1123.

b) V. Schellenberger, H. D. Jakubke, Angew Chem, Int. Ed. Engl. 1991, 30, 1437.

c) A. M. Klibanov. Chemtech. 1986, 16. 354.

d) L. E. S. Brink, J. Tramper, K. Ch. A. M. Luyben, K. V. Riet, Enzyme Microb. Technol. 1988, 10, 736.

e) Y. L. Khmelnitsky, A. V. Levashov. N. L. Klyachko, K. Martinek, Enzyme Microb. Technol. 1988, 10, 710.

f) M. N. Gupta, Eur. J. Biochem. 1992, 203, 25.

g) M. Waks, Proteins: Structure, Function, and Genetics, 1986, 1, 4.

h) J.S. Dordick, Biotechnol. Prog. 1992, 8, 259.; i) S. H. M.V. Erp, E.O. Kamenskaya, Y. L. Khmelnitsky, Eur. J. Biochem. 1991, 202, 379.

2). a) P. Kuhl, P. J. Halling, H. D. Jakubke, Tetrahydron Letters 1990, 31, 5213.

b) R. Affleck, Z .F. Xu, V. Suzawa, K. Focht, D. S. Clark, J.S. Dordick, Proc. Natl. Acad. Sci. USA, 1992, 89, 1100.

c) H. Kitaguchi, A. M. Klibanov, J. Amer. Chem. Soc. 1989, 111, 9272.

d) M. Stahl, M. O. Mansson, K. Mosbach, Biotech. Letters, 1990, 12(3), 161.

e) H. Kise, Bioorg. Chem. 1990, 18, 107.

f) H. Kise, Y. Tomiuchi, Biotech. Letters 1991, 13, 317.

g) T. Nagashima, A. Watanabe, H. Kise, Enzyme Microb. Technol., 1992, 14. 842.

h) Z. Zhong, J. J. C. Liu, L. M. Dinterman, M. A. J. Finkelman, T.W. Mueller, M. L. Rollence, M. Whitlow, C.H. Wong, J. Amer. Chem. Soc. 1991, 113, 683.

i) C. H. Wong, S. T. Chen, W. J. Hennen, J. A. Bibbs, Y. F. Wang, J. L. C. Liu, M. W. Pantoliano, M. Whitlow, P. N. Bryan, J. Amer. Chem. Soc. 1990, 112, 945.

j) R.S., Phillips, M.S. Matthews, E. Olson, R.L.V. Tersch, Enzyme microb. Technol. 1990, **12**, 731.

k) K. Chen, F.H. Arnold, Biotechnology, 1991, **9**, 1073.

3).   a) T. Sakurai, A. L. Margolin, A. J. Russell, A. M. Klibanov, J. Amer. Chem. Soc. 1988, **110**, 7236.

b) P.A. Fitzpatrick, A. M. Klibanov, J. Amer. Chem. Soc. 1991, **113**, 3166.

c) S. T. Chen, S. C. Hsiao, K. T. Wang, Bioorg. & Med. Chem. Letters. 1991, **1(9)**, 445.

d) S. T. Chen, S. Y. Chen, K. T. Wang, J. Org. Chem. 1992, **57**, 6960.

e) A.M. Klibanov, TIBS 1989, April, 141.

f) S. Riva, J. Chopineau, A.P.G. Kieboom, A.M. Klibanov, 1988, **110**, 584.

g) A.L. Margolin, D.F. Tai, A.M. Klibanov, J. Amer. Chem. Soc. 1987, **109**, 7885.

4).   S.T. Chen, S.H. Wu, K.T. Wang, Int. J. Peptpide Protein Res. 1991, **37**, 347.

5).   A typical procedure for removing water from the alcalase solution was as follows: the enzyme solution was suspended in an anhydrous alcohol by agitation, the resulting mixture was centrifuged (3000-4000 rpm) to separate the enzyme from the solvent, and the alcohol was removed by decantation. The procedure was repeated several times, and the enzyme was then transferred to a reaction flask for be further used. see reference 3-d.

6).   Alcalase was purchased from NOVO industrial (Denmark) as a brown liquid with a specific activity of 2.5 $AU.mL^{-1}$. (According to NOVO, one Anson-unit (AU) is the amount of enzyme which, under standard conditions, digests haemoglobin at an initial rate liberating per min an amount of TCA-soluble product, which gives the same color of phenol reagents as 1 mequiv of tyrosine. Thus1AU=1000U, 1U= 1 mmol of L-Tyr-OMe hydrolyzed per min).

7).   G. Merutka, W. Shalongo, E. Stellwagen, Biochem, 1991, **30**, 4245.

8).   a) S. T. Chen. S. Y. Chen, S. C. Hsiao, K. T. Wang. Biotech. Letters. 1991, **13(11)**. 773.

b) S. T. Chen, K. T. Wang, C. H. Wong, Chem. Commu. 1986, 1514.

c) S. T. Chen, S. C. Hsiao, A. J. Chiou, S. H. Wu, K. T. Wang, J. Chin. Chem. Soc. 1992, **39**, 91.